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## Using combined biomolecular methods to explore whale exploitation and social aggregation in hunter–gatherer–fisher society in Tierra del Fuego

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## ABSTRACT

Cetaceans were an important food and raw material resource for the South American hunter–gatherer–fisher (HGF) communities of Tierra del Fuego. Historic ethnographic evidence suggests that relatively mobile HGF groups came together in large numbers to exploit carcasses from individual cetacean stranding events. Substantial accumulations of whale bones within shell middens in the Lanashuaia locality of the Beagle Channel suggests that these social aggregation events may also have occurred in pre-historic periods. The difficulty in assigning taxonomic identifications to the fragmentary whale remains, however, made it difficult to explicitly test this hypothesis. Here, we applied two different biomolecular techniques, collagen peptide mass fingerprinting (ZooMS) and ancient mitochondrial DNA analysis to 42 archeological bone fragments from the Lanashuaia locality to provide accurate species identifications. There was a clear correspondence between ZooMS and DNA results, identifying five different cetacean species (Southern bottlenose, blue, humpback, right, and sei whale) as well as human and sea lion remains. The biomolecular results were not conclusively consistent with HGF social aggregation, revealing an unexpectedly diverse range of cetaceans within the Lanashuaia middens. However, the results could not fully refute the hypothesis that cetacean remains can be used as anthropic markers of aggregation events, as the observed species and haplotypes revealed potential shared exploitation of some whale resources between midden sites.

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## 1. Introduction

Cetaceans constituted a significant resource for hunter–gatherer–fisher (HGF) societies who lived at the uttermost tip of South America (Tierra del Fuego). Whale meat and blubber provided important nutritional sources, while whale bone was used as a raw material for tool manufacture (Borella, 2004; Piana, 2005; Scheinsohn, 2010). Fuegian societies, however, had not developed specific technology to hunt these mammals in the open sea; instead they primarily exploited individuals that drifted ashore (Gusinde, 1937). According to historical

sources when a beached whale was discovered, an aggregation event occurred, bringing people together to share the meat in a feast and to distribute other cetacean resources (Gusinde, 1937). Thus, whale remains provide a potential anthropic marker of aggregation processes in the HGF societies of Tierra del Fuego.

The presence of abundant cetacean remains at the sites of Lanashuaia I and II, two pre-contact shell middens located on the northern coast of Beagle Channel (Tierra del Fuego, South America) (Fig. 1), potentially reflects aggregation events focused on the exploitation of a single stranded animal. Fragmentation of the cetacean remains as a result of human activities precluded accurate taxonomic identifications based on traditional osteological techniques, thus an accurate assessment of the whale taxa present, and the minimum number of individuals (MNI) represented, could not be made. Consequently, in this study we applied biomolecular methods, based on collagen peptide mass fingerprinting (ZooMS) and ancient DNA, to achieve a twofold objective: a) to explore the cetacean species exploited by HGF groups;

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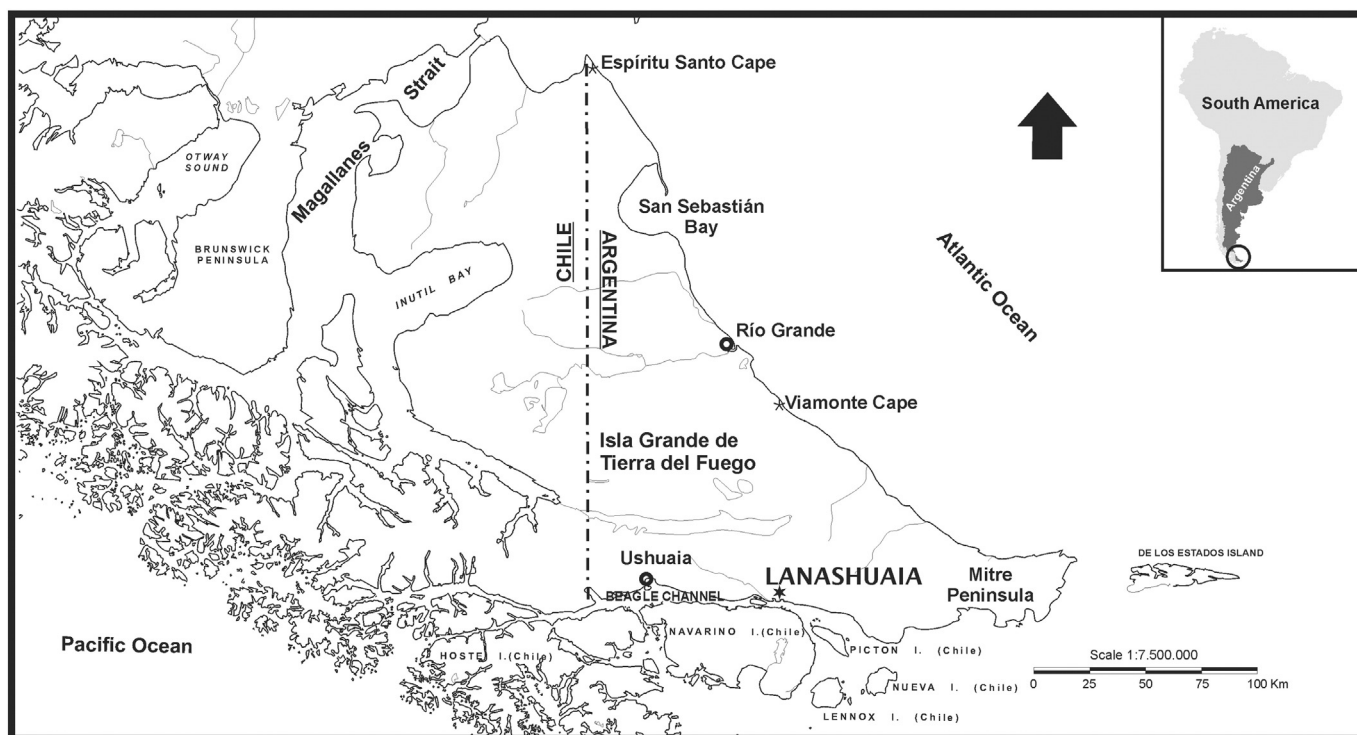


Fig. 1. Map of the Lanashuaia locality.

and b) to assess if whale bones are suitable material markers for identifying aggregation events in the region.

### 1.1. Aggregation in HGF societies of the Beagle Channel

Aggregations, or short-term gatherings of otherwise dispersed groups, are an important element in the social life of hunter-gatherers. Communal aggregations are an arena for dynamic social interaction and the promotion of cooperative activities (Conkey, 1980; Friesen, 1999; Hayden, 1993; Hofman, 1994; Kelly, 1995; Weniger, 1987). Ritual celebrations, economic cooperation, information exchange and exploitation of windfall resources (among others) bring people together in temporary concentrations. The situations under which an aggregation might occur are quite variable, and thus, the material correlates of an aggregation process are difficult to identify. The archeological record of the Beagle Channel in general, and the Lanashuaia site in particular, offers an extraordinary context to investigate aggregations as well as to explore novel methodologies in the archeology of HGF societies. The particular advantages of the pre-contact Lanashuaia site are: a) the excellent preservation of bone provided by shell midden layers due to the presence of calcium carbonate; b) the high-resolution stratigraphy of these kind of contexts; c) the rich archeological and ethnographic record of HGF societies of Tierra del Fuego, who called themselves Yámana or Yaghan during historical times (Gusinde, 1937, p. 986).

Using nautical technology, Yámana society developed an intensive exploitation of maritime resources (Gusinde, 1937, p. 986; Lothrop, 1928; Orquera et al., 2011). Ethnographic accounts of Yámana society from the 19–20C (Bridges, MS: 05-05-1872; 01-15-1872, 03-19-1872; Lothrop, 1928; Gusinde, 1937) indicate that sporadic episodes of social aggregation occurred following windfalls, including mass strandings of Fuegian sardines (*Sprattus fuegensis*, Jenyns), termed *aiacasi* in the Yámana language (Bridges, 1933), or when a whale came ashore. When a stranded cetacean was found, the discoverer was to send specific smoke signals to notify nearby people; if the signals were detected, a social aggregation event took place (Gusinde, 1937, p. 990; Martial, 1888, p. 181) (Fig. 2).

These events had unique importance to Yámana people for two reasons: they provided an exceptional accumulation of food and afforded the conditions to enhance social capital through a public demonstration of generosity (Briz et al., 2014; Gusinde, 1986, pp. 789–790). Nevertheless, although aggregation episodes are clearly documented in historical sources, their material remains are difficult to identify in the archeological record. This problem is compounded by the fact that there are over 500 shell middens recorded along almost 200 km of coast in the Beagle Channel area, representing more than 7000 years of occupation (and potentially re-occupation) (Orquera et al., 2011); therefore, identifying simultaneously occupied structures and shared social practice can be challenging. This project sought to identify the species of cetaceans exploited by HGF groups, and explore whether accurate biomolecular identification of cetacean taxa could provide a new anthropic marker of social aggregation in these pre-historic societies. The archeological expectations, according to the ethnographic accounts, would be that the majority of identified cetacean bones occurring within contemporaneous deposits within particular middens would belong to the same animal, and thus the same species.

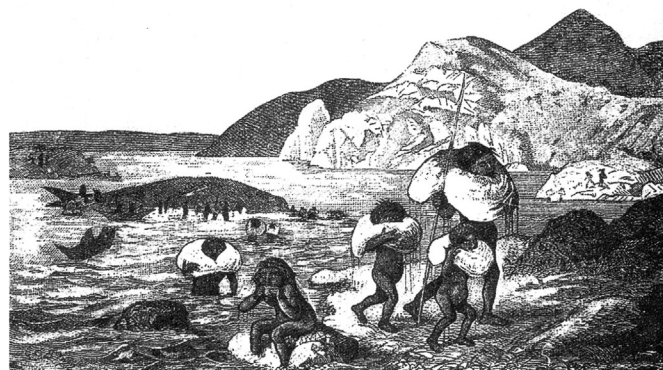


Fig. 2. Drawing of Yámana people butchering a whale (19C.), by Giacomo Bove (1883). (Legoupil, 2003, p. 133).

In order to accomplish these objectives, we focused on the Lanashuaia locality (also known as Lanushuaia) on the southern coast of Isla Grande de Tierra del Fuego ( $54^{\circ} 52,75''$  S– $67^{\circ} 16,49''$  W). Excavations carried out at Lanashuaia I shell midden had revealed 1023 whale bone fragments (Estevez et al., 2001), including a high frequency of bones morphologically identified as a young antarctic minke whale (*Balaenoptera bonaerensis*). This evidence (recovered from the base of the midden), combined with the remarkable alignment of several shell midden structures along the coastline, allowed us to test the hypothesis that these middens represent a single aggregation event (Piana et al., 2000, p. 461). Few whale bones from Lanashuaia I were accessible for biomolecular analysis, so we focused our research on Lanashuaia II a second, adjacent, and contemporaneously dated midden, which had been recently excavated and from which whale bones had also been identified (Fig. 3). The distance between both middens is approximately 6–8 m.

### 1.2. Lanashuaia I archeological context

Lanashuaia I (or Lanashuaia in preliminary publications), is a ring-shaped shell-midden, located on the isthmus that separates Cambaceres Interior and Cambaceres Exterior bays ( $54^{\circ} 52,75''$  S– $67^{\circ} 16,49''$  W) on the northern coast of the Beagle Channel. Over three fieldwork seasons (1995, 1996, under the direction of J. Estévez, E. Piana and A. Vila, and 2005, under the supervision of A. Álvarez, I. Briz and D. Zurro) an area of  $114\text{m}^2$  was excavated (Verdún, 2011). The shell midden most likely represents a single occupation, although three different episodes of human occupation are also possible but less likely (Verdún, 2011, p.46). Oxygen isotope analysis of archeological limpets (*Nacella magellanica*) at the site established that autumn/beginning of winter was the seasonal period of shell gathering (Colonese et al., 2011). The faunal remains present in the site include: fish (NISP = 1442); terrestrial (MNI = 2) and marine (MNI = 8) mammals; and birds (NISP = 435) (Colonese et al., 2011:250; Piana et al., 2000:464). As noted above, over 1000 fragments of whale bone were recovered from the site, as well as numerous bones morphologically identified as a young antarctic minke whale (*B. bonaerensis*) (Estevez et al., 2001). Radiocarbon dates from samples obtained from the base of the excavated area in 2005 offered the following results:  $1160 \pm 70\text{BP}$  (CNA301)  $1160 \pm 60\text{BP}$  (CNA302).

### 1.3. Lanashuaia II archeological context

Lanashuaia II is a ring-shaped shell-midden, located next to Lanashuaia I (Briz et al., 2009). Excavations were conducted over three seasons between 2009 and 2011 and covered an area of  $55\text{m}^2$  (approximately 80% of the total surface area of the ring-shaped feature); the annular structure and a large part of the intervening space between

Lanashuaia I and II were excavated during this period. The site is radiocarbon dated to between  $1155 \pm 40\text{BP}$  (CNA1056) to  $1385 \pm 25\text{BP}$  (CNA590), although there is no persuasive evidence to suggest that it is a multi-occupational site (i.e. no layers of humus between the layers of shells, which might indicate significant periods without human activities (Orquera and Piana, 1992)). These dates are comparable with those obtained for Lanashuaia I, suggesting that the sites were occupied contemporaneously, potentially as part of the same social aggregation event. Similar to Lanashuaia I, oxygen isotope analysis of *Nacella deaurata* and *Nacella magellanica* identified the primary shell gathering period as winter with sporadic collection in spring (Colonese et al., 2012). The faunal assemblage at Lanashuaia II is generally dominated by fish elements (NISP = 1280) (Gabriel, 2013), followed by bird (NISP = 568) and mammals remains (NISP = 530), the latter including 42 bones identified as Cetacea. The majority of the cetacean remains were highly fragmentary (ranging from 3 to 20 cm), modified by human action and, with the exception of two ribs, unidentifiable with regards to either skeletal element or species (Fig. 4). Preliminary morphological analysis by R.N.P. Goodall identified the more complete specimens as *B. bonaerensis* (antarctic minke) or potentially *B. borealis* (sei), through morphological comparison with the skeletal reference collection of the Acatushún Museum. Most of this evidence, like in the case of Lanashuaia I, was recovered from the base of the shell-midden, associated with the first occurrence of human activities on the site.

### 1.4. Issues in cetacean identification

Due to their large size and endangered status, whales exemplify and magnify common zooarcheological identification problems experienced in a range of different groups. First, there are relatively few comprehensive comparative collections of whale skeletons worldwide due to the considerable requirements of preparation, storage, curation and display. Moreover, the morphology of many whale bones is similar, making it challenging to accurately identify bone fragments to species. This latter issue is compounded by deliberate fragmentation due to human butchery or artifact creation, as well as the overall friability of archeological whale bone, producing a large quantity of visually unidentifiable fragments. Biomolecular methods can provide far more accurate identifications than morphological analysis. Here we compared the accuracy and robusticity of two biomolecular identification methods: ZooMS (Zooarcheology by Mass Spectrometry) and ancient DNA analysis.

The ZooMS method involves the peptide mass fingerprinting (PMF) of collagen, the main protein constituent of bone. The relatively slow rate of evolution within the collagen triple helix means that collagen molecules are variable enough to discriminate between mammalian genera through PMF, but also sufficiently similar to map differences across more widely-dispersed taxonomic groups (Buckley et al.,

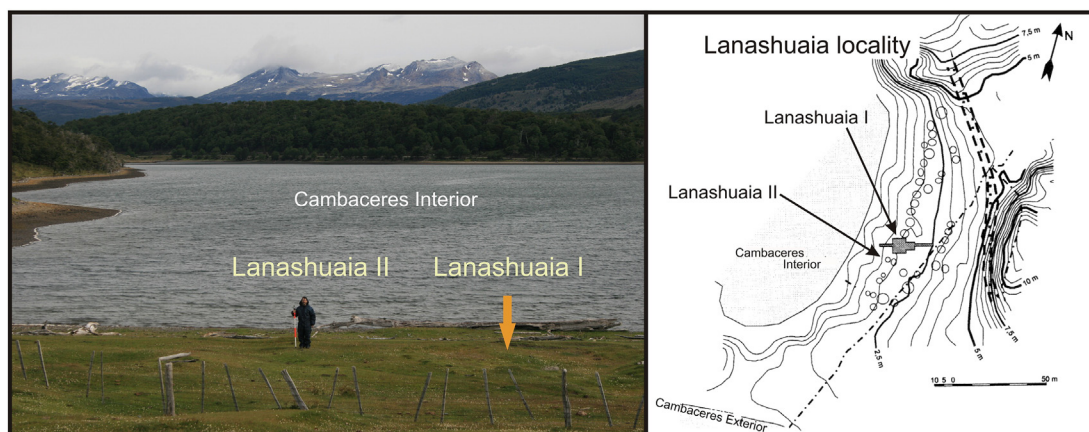


Fig. 3. Locations of Lanashuaia I and II in the isthmus between Cambaceres Interior and Cambaceres Exterior bays. (Map from Piana et al., 2000: 465).





Fig. 4. Fragment of a cetacean rib recovered from Lanashuaia II.

2009). Using PMF, the ZooMS method has already been successfully applied to discriminate between archeological cetacean genera within the North Atlantic (Buckley et al., 2014), but has not yet been applied to southern hemisphere species. DNA analysis has a far longer history than ZooMS, and mitochondrial DNA (mtDNA) analysis has been the more traditional approach for taxonomic identifications of archeological marine species in general (Cannon et al., 2011; Foote et al., 2012; Moss et al., 2006) and fragmentary cetacean remains in particular (Foote et al., 2012; Losey and Yang, 2007; Rastogi et al., 2004; Yang and Speller, 2006).

In this study, we applied both ZooMS and mtDNA analysis to identify the species of cetaceans exploited at Lanashuaia and their concordance with material correlates of an aggregation event. Based on historical sources, correlates would include the predominant exploitation of a single cetacean species, evidence for the contemporaneity of multiple middens (e.g. Lanashuaia I and II), the identification of the same cetacean species in both middens, and shared social practices developed during the event (for example, sharing different products of hunting activity between both shell-middens or lithic working evidenced by lithic refitting, among others). While ZooMS and mtDNA analysis do not have the diagnostic precision to confirm the exploitation of the same whale individual, the species and haplotype data can be used to assess whether whale remains represent suitable material markers for identifying aggregation events in the region.

## 2. Materials and methods

### 2.1. Archeological samples

Fragmentary bone samples morphologically identified as cetaceans were selected for identification using ZooMS and ancient DNA techniques (Table 1). Thirty-eight samples were selected from 17 different layers within Lanashuaia II. These were compared with five available bone samples from Lanashuaia I, two of which were thought to come from the same rib.

### 2.2. Museum reference material

Seven bone samples from cetacean species native to Tierra del Fuego were provided for comparison by the Acatushún Museum in Tierra del Fuego, including: two samples of *Balaenoptera borealis* (sei), two *Balaenoptera acutorostrata* (common minke), two *Balaenoptera bonaerensis* (Antarctic minke) and one *Eubalaena australis* (Southern right whale).

The reference collection at the Acatushún Museum is created from specimens beached in the Argentinean sector of Isla Grande de Tierra del Fuego, as well as some sporadic specimens from the Chilean sector.

In the case of the Argentinean samples, taxonomic identifications are accomplished by the Acatushún Museum staff applying traditional morphological methods (e.g. comparison with pre-existing osteological collections or with an anatomical atlas). Where complete skeletons are available, taxonomic identifications take into account the growth patterns of the skull (e.g. length of basal condyle, length of the face, width of the basis of the face, maximum width of the nostrils). In the case of an incomplete or fragmentary specimen, the identification of diagnostic bone elements is crucial for a potential species identification, particularly for the different vertebrae types. From the skull to the tail there is significant taxonomic variation in the height of the spinous processes and length of transverse processes, as well as width, length and morphology of vertebral bodies and intervertebral disks. These morphological variations in cetacean vertebrae are directly related to swimming technique and trophic habitat of every species. The Acatushún collection includes partial and entire specimens of *B. bonaerensis*, *B. acutorostrata*, and *B. borealis*, recovered from stranding episodes in Tierra del Fuego.

### 2.3. ZooMS identifications

The 38 samples from Lanashuaia II and seven museum reference samples were first subjected to ZooMS analysis using a combination of non-destructive (Korsow-Richter et al., 2011) and destructive (Buckley et al., 2009) collagen extraction techniques. Insufficient collagen yields were obtained for the majority of the samples using the non-destructive approach. As a result all samples were then prepared for analysis using a more aggressive demineralization technique, followed by ultrafiltration. The results presented here include the two samples successfully extracted using the non-destructive technique (7264 and 7269) and the remaining samples prepared using the destructive method.

#### 2.3.1. Non-destructive method

Approximately 10 mg of bone chips or powder from each sample was placed into an eppendorf tube with 100 µl of 50 mM ammonium bicarbonate (AmBic) solution (pH 8.0) and left at room temperature overnight. Following centrifugation, the supernatant was discarded, and samples were re-incubated in a fresh 100 µl of AmBic solution at 65 °C for one hour; 50 µl of the second supernatant was incubated with 0.4 µg of trypsin, acidified to 0.1% trifluoroacetic acid (TFA), and purified using a 100 µl C<sub>18</sub> resin ZipTip® pipette tip (EMD Millipore). Conditioning and eluting solutions for the C-18 tips were composed of 50% acetonitrile and 0.1% TFA, while 0.1% TFA was used for the lower hydrophobicity buffer. To ensure maximal collagen retrieval, the sample was transferred completely through the tip five times and eluted in 50 µl.

#### 2.3.2. Destructive method: demineralization and ultrafiltration

For each sample between 10 and 30 mg of bone (chips or powder) was placed in eppendorf tubes and demineralized through immersion in 250 µl of 0.6 M hydrochloric acid for 48 h to up to two weeks at room temperature. Following centrifugation, the supernatant was discarded and samples were incubated in an additional 250 µl of 0.6 M HCl for three hours at 65 °C to gelatinise the collagen. Following gelatinisation, the collagen was ultrafiltered using Amicon Ultra-4 centrifugal filter units (30,000NMWL, EMD Millipore) to remove impurities. The supernatant was concentrated to approximately 100 µl, washed three times with 200 µl AmBic solution, and concentrated to a final volume of 50 µl. Trypsin digestion and collagen purification was conducted following the method listed above.

#### 2.3.3. MS, spectral processing, and taxonomic identifications

For mass spectrometry, 1 µl of extract was mixed with an equal volume of matrix (1 µl of α-cyano-hydroxycinnamic acid) and spotted onto a 384 spot target plate, along with calibration standards. Each

**Table 1**

Provenience and identification information for museum and archeological specimens.

Lab code	Loc.	mg bone powder <sup>a</sup>	# of spectra <sup>b</sup>	Morphological ID	ZooMS ID	DNA ID Cytb <sup>c</sup>	DNA ID D-loop <sup>c</sup>
<i>Acatuashuín museum</i>							
6737	RNP2362	–	6	<i>B. borealis</i> (Sei)	Sei	Not tested	
6738	RNP1605	9.3	9	<i>B. borealis</i> (Sei)	Sei	<i>B. borealis</i>	<i>B. borealis</i>
6739	RNP1027	31.7	9	<i>B. acutorostrata</i> (Common minke)	Minke	<i>B. acutorostrata/bonaerensis</i>	<i>B. bonaerensis</i>
6740	RNP1047	–	9	<i>B. acutorostrata</i> (Common minke)	Balaenoptera (sei/minke)	Not tested	
6741	RNP2333	27.2	9	<i>B. bonaerensis</i> (Antarctic minke)	Minke	<i>B. acutorostrata/bonaerensis</i>	<i>B. acutorostrata/bonaerensis</i>
6742	RNP1296	–	9	<i>B. bonaerensis</i> (Antarctic minke)	Minke	Not tested	
6743	RNP127	1.5	9	<i>E. australis</i> (S. right whale)	Balaenoptera (sei/minke)	<i>B. acutorostrata/bonaerensis</i>	<i>B. acutorostrata/bonaerensis</i>
<i>Lanashuaia I layer</i>							
7440	B800	12.3		Cetacean	Not tested	<i>B. musculus</i>	<i>B. musculus</i> (Hap1)
7481	C200	17.9		Cetacean	Not tested	<i>M. novaeangliae</i>	<i>M. novaeangliae</i> (Hap2)
7678	C321	25.5		Cetacean	Not tested	<i>M. novaeangliae</i>	<i>M. novaeangliae</i> (Hap3)
7869a <sup>1</sup>	B1400	16.3		Cetacean (rib)	Not tested	<i>E. australis</i>	<i>E. australis</i>
7869b <sup>1</sup>	B1400	21.8		Cetacean (rib)	Not tested	<i>E. australis</i>	<i>E. australis</i>
<i>Lanashuaia II layer</i>							
7261	C1080	107/4.0	9	Cetacean	Right/bowhead	<i>E. australis</i>	
7262	C1010	71.5	6	Pinniped	No ID	Fail	
7263	B10	1.0	6	Pinniped	No ID	Fail	
7264	C1130	22.4	3	Cetacean	Right/bowhead	<i>E. australis</i>	
7265	B10	25.7	6	Cetacean	Cetacean	<i>M. novaeangliae</i>	
7266	C1050	32.1	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7267	B490	25	9	Cetacean	Probable humpback	<i>M. novaeangliae</i>	
7268	B510	18	6	Cetacean	Balaenoptera (sei/minke)	<i>B. borealis</i>	<i>B. borealis</i>
7269	C1050	27.8	6	Cetacean	Cetacean	<i>B. musculus</i>	<i>B. musculus</i> (Hap1)
7270	B10	17.8	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7271	B400	57.1	9	Cetacean	Probable Humpback	<i>M. novaeangliae</i>	
7272	C1080	6.5	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7273	T2	2.0	6	Cetacean	Right/bowhead	<i>E. australis</i>	
7274	B630	10.0	6	Cetacean	Right/bowhead	<i>E. australis</i>	
7275	B620	1.0	6	Cetacean	Right/bowhead	<i>E. australis</i>	Fail
7276	B10	30.6	6	Bone	No ID	Fail	
7277	C1060	1.4	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7278	C1050	23	6	Cetacean	Blue	<i>B. musculus</i>	<i>B. musculus</i> (Hap1)
7279	B10	3.0	6	Cetacean	Probable Blue	<i>B. musculus</i>	Fail
7280	C1050	4.5	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7281	T4	35	6	Cetacean	Right/bowhead	<i>E. australis</i>	<i>E. australis</i>
7282	C1080	48/1.5	9	Cetacean	Beaked whale	<i>H. planifrons</i>	Fail
7283	C1020	46.6	9	Cetacean	Humpback	<i>M. novaeangliae</i>	
7284	C20	25.2	3	Cetacean	Cetacean	<i>B. musculus</i>	Fail
7285	B10	20/1.7	6	Cetacean (rib)	Right/bowhead	<i>E. australis</i>	<i>E. australis</i>
7286	C1080	11	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7287	B420	98/1.1	9	Cetacean	Humpback	<i>M. novaeangliae</i>	<i>M. novaeangliae</i> (Hap4)
7288	C1050	20.9	6	Cetacean	Primate/human	Fail	
7289	C1050	18.8	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7290	C1050	7.8	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7291	B10	42.1	9	Cetacean	Fur seal/sea lion	<i>Otaria flavescens</i>	
7292	B10	39	9	Cetacean	Blue whale	<i>B. musculus</i>	<i>B. musculus</i> (Hap1)
7293	B10	28/1.3	3	Cetacean	Blue whale	<i>B. musculus</i>	<i>B. musculus</i> (Hap1)
7294	B20	44.9	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7295	B620	8.0	6	Cetacean	Right/bowhead	<i>E. australis</i>	
7296	B10	18.3	6	Cetacean	Beaked whale	<i>H. planifrons</i>	
7297	C1050	42.6	6	Cetacean	Mysticeti	<i>M. novaeangliae</i>	
7298	C20	26/14.6	6	Cetacean	Cetacean	<i>B. musculus</i>	<i>B. musculus</i> (Hap1)

<sup>a</sup> Indicates mg of bone powder used in initial/repeat DNA extractions.<sup>b</sup> Number of quality spectra used for ZooMS identification.<sup>c</sup> fail indicates no PCR amplification.<sup>1</sup> Radiocarbon date: 1780 ± 40 (Beta 287,372).

sample was spotted in triplicate, and was run on a Bruker ultraflex III Matrix-assisted laser desorption/ionization time of flight (MALDI TOF/TOF) mass spectrometer with a Nd:YAG smartbeam laser. A SNAP averagine algorithm was used to obtain monoisotopic masses (C 4.9384, N 1.3577, O 1.4773, S 0.0417, H 7.7583). With replications and re-spotting, a total of 303 individual spectra were collected and analyzed (SI Material I).

Spectra were visually inspected using the mMass software (Strohm et al., 2008) and poor quality spectra (i.e. with low signal to noise ratios, or few to no discrete peaks) were eliminated from the dataset. Spectra from replicates of the same sample were averaged, and compared to the list of *m/z* markers for marine mammals presented in (Buckley et al., 2014) and

for non-marine species presented in (Kirby et al., 2013) and (Buckley and Kansa, 2011) (see SI Table 1 for list of designated peptide markers used to identify each taxa). Taxonomic identifications were assigned at the most conservative level of identification (genus, or family level) based on the presence of unambiguous *m/z* markers.

## 2.4. Ancient DNA analysis

### 2.4.1. Sample preparation, extraction and amplification

DNA analysis was then conducted on the 38 archeological bone samples from Lanashuaia II, five available bone samples from Lanashuaia I, and on the four museum reference specimens with sufficient materials

for analysis. Preparation and DNA extraction of the archeological bone samples were conducted in the ancient DNA laboratory at University of York, which follows strict protocols for contamination control and detection, including positive pressure, the use of protective clothing, UV sources for workspace decontamination, and laminar flow hoods for extraction and PCR-set-up. Museum specimens were processed in a separate laboratory space. Less than 50 mg of bone powder was weighed out (Table 1) and UV irradiated for 30 min; DNA was extracted using a silica spin column protocol (Yang et al., 1998) modified as reported in Dabney et al. (2013), and DNA was eluted in 50  $\mu$ l of TET buffer. Initial PCR amplifications targeted a 182 base pair (bp) fragment of the cytochrome b (cytb) mitochondrial gene which has been demonstrated to successfully distinguish cetacean species (Yang and Speller, 2006). A subset of the samples was also amplified with primers targeting approximately 237 bp of cetacean mtDNA D-loop (Yang and Speller, 2006) or 180 bp of pinniped cytb (Moss et al., 2006) to confirm ambiguous species designations, or in case of blue whale, to provide additional information concerning the MNI represented by the bone samples. PCR reactions and cycling conditions followed Speller et al. (2012) and PCR products were sequenced using forward and/or reverse primers at Eurofins Genomics, Ebersberg, Germany.

#### 2.4.2. Sequence analysis and species identifications

The obtained sequences were visually edited using the ChromasPro software ([www.technelysium.com.au](http://www.technelysium.com.au)), truncated to remove primer sequences. Edited sequences were compared with published references through the GenBank BLAST application (<http://www.ncbi.nlm.nih.gov/BLAST/>). Multiple alignments of the ancient sequences and published cetacean sequences were conducted using ClustalW (Thompson et al., 1994), through BioEdit (<http://www.mbio.ncsu.edu/BioEdit>). Species identifications were assigned to a sample only if it was identical or very similar to the published reference sequences (99%), and if no other evidence, including reproducibility tests, additional sequencing of the same sample indicated a different species. Species identities were confirmed through 'DNA Surveillance', a web-based program which aligns a user-submitted sequence against a comprehensive set of validated cetacean reference sequences to provide a robust identification for whales,

dolphins and porpoises (Baker et al., 2003). Fifty-nine sequences were uploaded to the Genetic Sequence Database at the National Center for Biotechnical Information (NCBI) (GenBank ID: KR087065–KR087123); a summary of haplotype data is presented in SI Table 2.

As DNA analysis was conducted after the ZooMS analysis, some samples had only miniscule quantities of bone powder available for analysis (<2 mg). A subset of samples with ample bone powder underwent repeat extractions and PCR amplifications using <3 mg of bone powder to assess whether consistent DNA species identifications could be obtained from minute amounts of bone powder (Table 1).

### 3. Results

#### 3.1. ZooMS identifications

Following analysis of the PMF spectra, taxonomic identifications could be assigned to the seven museum species and 35 of the 38 archeological samples (Table 1, SI Table 3 for complete table of identified peptide markers). In the case of the museum species, ZooMS identifications were consistent with the museum taxonomic identifications in six of the seven samples. ZooMS correctly identified the two *B. borealis* samples as sei, and the two *B. bonaerensis* and two *B. acutorostrata* samples were identified either as minke whales ( $n = 3$ ) or to the genus *Balenoptera* ( $n = 1$ ). The museum sample 6743, identified morphologically as *E. australis* (Southern right whale) was identified by ZooMS as '*Balenoptera*' (either minke or sei) though species identification was not possible due to absence of peptide C at either 1550 or 1566 (Fig. 5).

Of the 35 Lanashuaia II archeological samples that produced spectra with sufficient peaks for taxonomic identification, 11 samples were identified as beaked whales (bottlenose whales), eight samples as right/bowhead, four as humpback, and four as blue whale (Fig. 6). Due to some ambiguous markers, some samples could not be assigned to the species/genus level, including one sample identified to the genus *Balenoptera* (either minke or sei), one to the suborder *Mysticeti*, and four to the order *Cetacea*. Additionally, two samples were identified as taxa other than cetaceans: one probable human (SI Fig. 1), and one fur seal/sea lion.

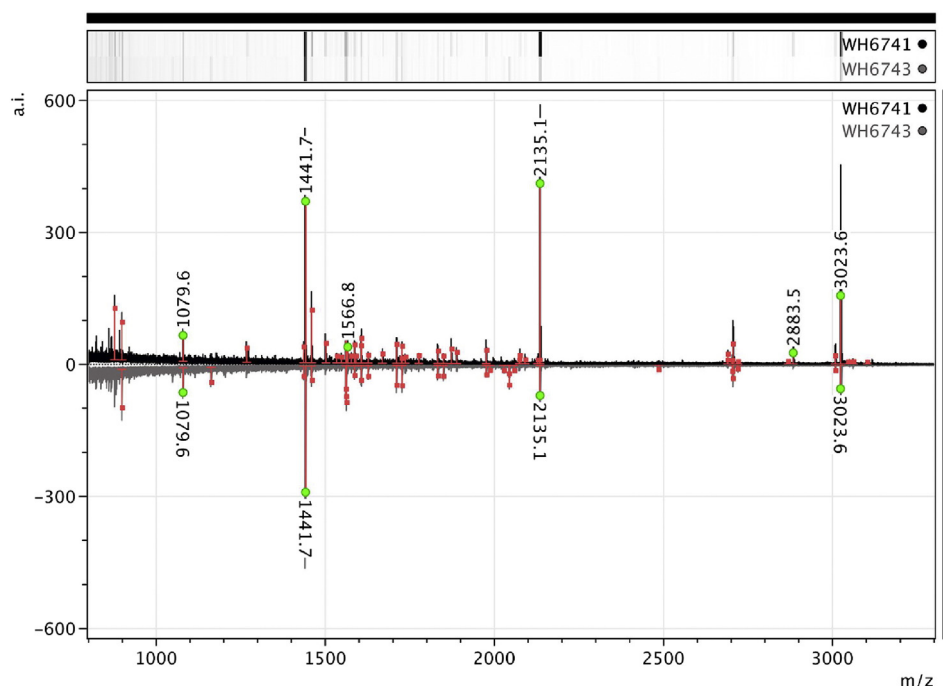


Fig. 5. MALDI-TOF spectra of 6741 (minke, top) and 6743 (bottom) with whale specific markers noted.



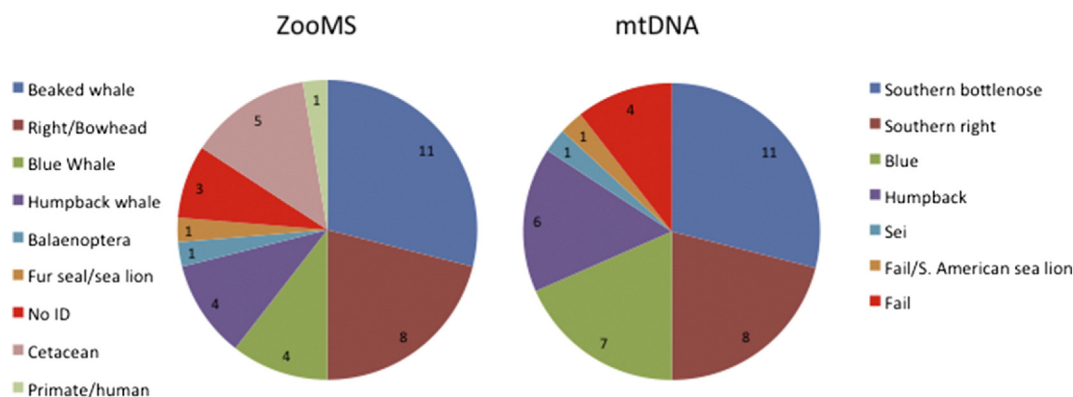


Fig. 6. Comparison of species identification results of Lanashuaia II samples using ZooMS and mtDNA analysis.

### 3.2. DNA identifications

Cytb and D-loops sequences were recovered for all four museum samples with sufficient bone powder for DNA analysis. In three of the four samples, the mtDNA and morphological identifications were consistent. Similar to the ZooMS results, both cytb and D-loop sequences were consistent with the morphological identifications of the sei and minke whales, but identified 6743, the *E. australis* sample as minke whale. Common minke (*B. acutorostrata*) and Antarctic minke (*B. bonaerensis*) can be challenging to differentiate based on relatively short mtDNA fragments, and the cytb and D-loop sequences obtained in the study matched with 100% identity to both species. Accurate mtDNA differentiation of the two species is compounded by observations of hybridization and backcrossing in modern individuals (Glover et al., 2010).

Taxonomic identifications were assigned to 34 of the 38 archeological Lanashuaia II samples, and all five Lanashuaia I samples using ancient mtDNA sequences. Five samples failed to amplify using the whale-specific cytb primers: these included the three samples that failed to produce diagnostic peaks using ZooMS (7262, 7263, 7276) as well as the two non-whale samples (7288: probably human; 7288: fur seal/sea lion). Of the 33 successfully amplified Lanashuaia II samples, DNA identified 11 as Southern bottlenose whale (*Hyperoodon planifrons*), eight Southern right (*E. australis*), six humpback (*Megaptera novaeangliae*), seven blue (*Balaenoptera musculus*), and one sei whale (*B. borealis*) (Table 1). Subsequent amplification of sample 7288 with pinniped cytb primers identified the sample as South American sea lion (*Otaria flavescens*). At Lanashuaia I, DNA identified two Southern right whale (thought to be from the same rib), two humpback, and one blue whale (Table 1; Fig. 7). All of the respective whale species from the two Lanashuaia II shared the same cytb haplotype. At Lanashuaia I, however, the two humpbacks displayed different cytb haplotypes, one of which matched the samples from Lanashuaia II.

To further explore MNI and shared haplotypes between the sites, a subset of 17 samples were amplified using a longer mtDNA D-loop primer set, including all samples from Lanashuaia I, one of each whale species from Lanashuaia II and all of the blue whale samples. In all cases, the D-loop sequence confirmed the cytb identification. Within the blue whale samples, six of the eight blue whale samples were successfully amplified using the D-loop primer set, all of which yielded identical D-loop haplotypes, shared across the two middens (MNI = 1). The two humpback whales from Lanashuaia I also had different D-loop haplotypes, neither of which were shared with the Lanashuaia II site (MNI = 3).

Four samples of different species were re-extracted using <3 mg of bone powder to ascertain the reliability of DNA amplifications from minute sample sizes. For the blue, right and humpback whale, successful cytb and D-loop amplifications were obtained from re-extracted samples, yielding identical sequences to initial extractions undertaken

with >20 mg of bone powder. For the Southern bottlenose whale, both the original and repeat DNA extract failed to amplify using the D-loop primers (likely due to primer incompatibility), but produced identical cytb sequences. No amplifications were observed in any of the blank extractions or negative controls for either initial or repeat extractions and amplifications.

## 4. Discussion

### 4.1. Molecular species identifications – methodological implications

#### 4.1.1. Authenticity of the molecular identifications

Due to the typically low quality and quantity of endogenous DNA template in archeological bones, and the necessity for sensitive PCR amplification protocols, ancient DNA analysis is particularly vulnerable to contamination from modern sources compared to collagen-based approaches. Several lines of evidence can be used to secure the DNA identifications in this study, including: (i) the use of dedicated ancient DNA facilities equipped with UV filtered ventilation, positive airflow, and bench UV lights; (ii) the separation of pre- and post-PCR workspaces; and (iii) the absence of amplification in all of the blank extracts and PCR negative controls. Multiple species were identified within each extraction batch, and repeat extractions and amplifications consistently yielded the same sequences, even when using small quantities of bone powder. All of the identified species are in correspondence with the biogeography of whale species within the southern hemisphere and around Tierra del Fuego in particular (Watson, 1985). Moreover, the ZooMS and aDNA results produced consistent taxonomic identifications, even when in disagreement with the morphological identification of one museum reference sample.

#### 4.1.2. Museum reference samples

Seven museum cetacean samples were analyzed to test the accuracy of the biomolecular identification methods. The morphological, ZooMS and DNA identifications were in accordance with the sei, common minke whale and Antarctic minke specimens, although the three methods produced identifications at different taxonomic levels. In the case of the sample morphologically identified as Southern right whale (*E. australis*), ZooMS identified this sample to the genus *Balaenoptera* and both the cytb and D-loop sequences matched with minke whale. This discrepancy between molecular and morphological identifications highlights the difficulty in identifying fragmentary cetacean remains, and the opportunity offered by molecular methods to improve the quality of museum specimen identifications.

#### 4.1.3. Correspondence of ZooMS and DNA identification methods

The application of two biomolecular approaches to the same archeological assemblage highlights the relative advantages and limitations of both techniques. The success rate for molecular identifications

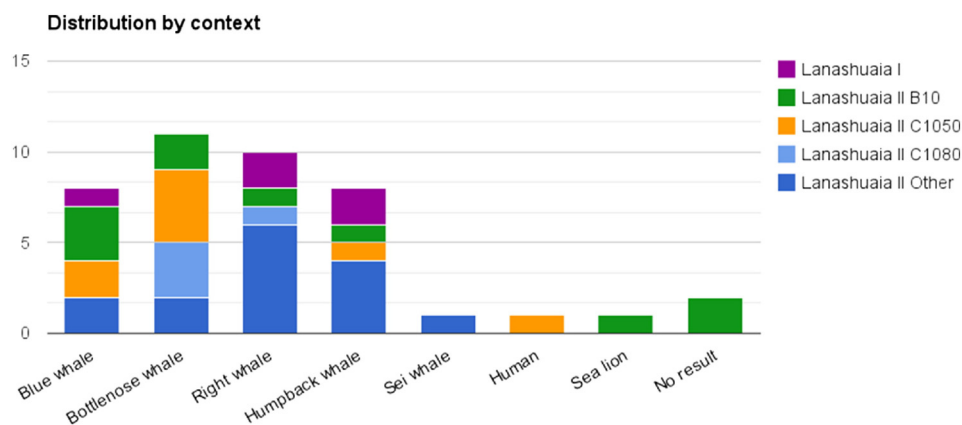


Fig. 7. Distribution of species within contexts at Lanashuaia I and II.

of the Lanashuaia II samples were comparable for ZooMS (35/38) and DNA (33/38 using cytb cetacean primers), with a slightly higher success rate for the collagen-based identification method. The advantage of ZooMS lay in its ability to identify bones without prior knowledge of family order, demonstrated in this study by the identification of pinniped and human bone samples which failed initial mtDNA amplification due to primer incompatibility. At Lanashuaia II, ZooMS could identify blue and humpback whales to the species level, and the sei whale to the genus level. While collagen PMF cannot distinguish between the right and bowhead, the southern hemisphere location of Lanashuaia virtually eliminates the possibility of bowhead whale at the site - an advantage over northern hemisphere archeological sites where both species may be present. Likewise, the only right whales species expected at this site would be Southern right whale (*E. australis*). Nevertheless, due to ambiguities in the spectra, as well as the relatively slow evolution of the collagen sequence, ZooMS cannot always provide the same level of taxonomic accuracy as mtDNA analysis. In this study, six samples lacked the necessary *m/z* peaks to make a confident genus or species level identification, and samples within the Family *Hyperoodontinae* (beaked whales) could not be identified to the species level.

In contrast, DNA could identify the great majority of samples to the species level, failing only to confidently distinguish between southern hemisphere Antarctic minke (*B. bonaerensis*) and the cosmopolitan common minke (*B. acutorostrata*). These two species share mtDNA haplotypes, and can hybridize and back-cross (Glover et al., 2013), rendering a purely mtDNA-based identification problematic. Moreover, DNA could distinguish some individuals on the bases of distinct haplotypes, indicating for example, an MNI of one for the blue whale, and three for the humpback whales.

In general, the majority of samples displayed well preserved biomolecules. The amplification of replicable mtDNA sequences up to 230 bp in length from as little as 1 mg of bone powder suggests excellent biomolecular preservation at Lanashuaia. Interestingly, the three samples that failed to produce ZooMS spectra with identifiable peaks also failed aDNA analysis. Collagen is typically a more robust biomolecule than DNA, often surviving relatively intact for millennia, even in low latitudes where DNA preservation is poor (Welker et al., 2015). Thus, the concurrence in success rate for the two methods suggests a potential role for ZooMS as a screening method for overall biomolecular preservation. As noted in von Holstein et al. (2014), ZooMS provides a logical screening method ahead of DNA, to provide an initial taxonomic identification, and screen for overall biomolecular preservation. Additionally, even higher order taxonomic ZooMS identifications are useful for validating ancient DNA results which can be more susceptible to contamination from modern DNA sources or previously amplified PCR products.

#### 4.2. Correspondence with whale biogeography and stranding records

The taxa recovered at Lanashuaia not only offer insight into the range of species exploited by prehistoric HGF groups, but also pre-contact evidence for cetacean presence and/or stranding in this region. The presence of whales in the Fuegian region is related to the Antarctic Circumpolar Current (ACC). This current is cold and rich in nutrients and nourishment, and, consequently, attracts a high number of Austral species and/or migrants to Antarctic area. The islands of Tierra del Fuego are the only portion of land within the ACC. As a result, Fuegian beaches may accumulate both stranded individuals, as well as carcasses from animals which died at sea (e.g. from diseases, old age, predators or collisions with ships) and which were carried by the ACC to the island shores.

For the Tierra del Fuego region, a comprehensive systematic record of strandings was only begun in 1975 by Natalie P. Goodall, in cooperation with the Department of Biology of the Centro Austral de Investigaciones Científicas-CADIC. From 2001, this stranding information has also been collected by the Acatushún Museum staff, and includes 31 species (*Delphinidae*, *Phocoenidae*, *Ziphiidae* and *Balaenidae*) and more than 2000 specimens.

The most characteristic stranded whales in Tierra del Fuego are *B. acutorostrata* ssp. (Dwarf minke whale; N = 20) and *B. bonaerensis* (Antarctic minke whale; N = 9). The geographical distribution of these strandings are: Northern Atlantic Coast (11), Central Atlantic Coast (6) and Beagle Channel (7). In this last case, the strandings of whales are mainly produced by the attacks of killer whales (*Orcinus orca*). According to the Acatushún records, other more frequently stranding species in the Fuegian coasts are *Cephalorhynchus commersonii* (Commerson's dolphin) and *Phocoena dioptrica* (Spectacled porpoise) due to their coastal behavior and the existence of artisanal fishing. In the case of *C. commersonii*, the Acatushún records report 965 strandings, especially in the central area of the Atlantic coast of Isla Grande de Tierra del Fuego, including the areas of Río Grande, Punta Popper, Cabo Peñas, Punta María and Cabo Viamonte (450 strandings from 1975) and in the Northern area of this coast: Bahía San Sebastián, Península Páramo and Cabo Espíritu Santo (around 400 strandings). In the case of *P. dioptrica*, 365 strandings are recorded in the same areas of the *Cephalorhynchus commersonii*: 176 in the case of the same Northern area (Bahía San Sebastián-Cabo Espíritu Santo) and 126 in the central area (Río Grande-Cabo Viamonte). Also for both Atlantic Coast areas, strandings of *Lagenorhynchus australis* (Peale's dolphin; N = 77) and *Lissodelphis peronii* (Southern right whale dolphin; N = 70) are not uncommon. An interesting example of stranding in the Atlantic Coast and the Beagle Channel is the *Globicephala melas* (Long-finned pilot whale). Their strandings (231 from 1975) are very sporadic, but often occur in groups.



Interestingly, none of these frequently stranding species were identified in the Lanashuaia remains analyzed in this study. The biomolecular results, however, are still compatible with modern stranding records for the majority of the identified species. The most abundant species at the site, the Southern bottlenose has a widespread distribution and is known to be among the most abundant of the *Ziphiidae* in the region (MacLeod et al., 2005). Tierra del Fuego and the Malvinas/Falkland Islands form foci for concentrations of beaked whales, and among them Southern bottlenose whales form one of the four most common species recorded in strandings from these areas (Otley, 2012), along with *Ziphius cavirostris* (Cuvier's beaked whale), *Mesoplodon layardii* (Layard's beaked whale), and *Mesoplodon grayi* (Gray's beaked whale). The presence of only the Southern bottlenose at the Lanashuaia locality is intriguing; more refined nuclear DNA analysis, however, would be needed to ascertain whether one or multiple individuals were present at the site.

An important summer feeding ground for humpback whales has been identified in the Straits of Magellan (Acevedo et al., 2007) and others are known around the Antarctic Peninsula. While humpbacks do not strand frequently (Watson, 1985), records suggest that strandings do occur along the migratory routes of the species (Moura et al., 2013). Humpbacks generally follow coastal migration routes (Watson, 1985), and recent studies have shown that those feeding off the Antarctic Peninsula migrate northward along the western coast of South America, to breeding grounds on the North-West Coast (Stevick et al., 2004), thus passing Fuegian Islands, which also lie close to the feeding ground in the Straits of Magellan.

Likewise, between June and December each year the waters near Península Valdés on the Argentinean coast play host to congregations of Southern right whales, who collect in the area to calve. The whales follow their annual migrations from cooler summer feeding grounds possibly as far south as the Antarctic peninsula (NOAA, 2012), past Tierra del Fuego, sometimes entering the Beagle Channel, and on to Península Valdés. Strandings of this species are not particularly common, and only five strandings (and six sightings) were recorded by Goodall and Galeazzi (1986) for Tierra del Fuego.

Only a single sei whale was identified at the site, which is consistent with stranding records for this species. In the southern hemisphere sei whale populations span the subtropical regions to the Antarctic convergence. Although historic whaling records indicate formerly high numbers of sei whales along the Argentinean coasts (Iñiguez et al., 2010; White, 2002), strandings of baleen whales in general are uncommon in this area (Leonardi et al., 2011, p. 177). This may reflect a wider phenomenon, and records from the northern and southern hemisphere suggest that sei whales rarely strand (Brabyn, 1991; NHM, 2011).

The most unusual discovery at Lanashuaia was the presence of blue whale. Modern day studies of blue whale distributions indicate that this species is rare in the south-western South Atlantic (Otley, 2012) and although the exact numbers of pre-whaling populations are unknown, general figures suggest that today's population represents only a small percentage of pre-commercial whaling numbers (Branch et al., 2004). According to Goodall et al. (2008), there are no records of blue whales stranding in Tierra del Fuego, and two possible strandings of blue whales in the Malvinas/Falkland Islands remain unverified (Otley, 2012). While recent figures suggest the species are rare in the southern hemisphere, their rarity in the Malvinas/Falklands and Tierra del Fuego area may also reflect habitat preferences and the relatively shallow depths of the Patagonian Shelf may lead the species to avoid this area in favor of deeper waters. Whether the rarity of blue whale in the south-western South Atlantic is ecological or a consequence of overexploitation, their presence at Lanashuaia in the first century CE brings to mind the words of Waite Hockin Stirling who served as a missionary at Ushuaia:

*"But there can be no doubt that the health of the native population has seriously deteriorated of late years, and this, among other causes, owing*

*to the scarcity of the whale and seal. These creatures used to supply the Fuegian native with food and clothing suitable to his wants. The destruction, however, of these has been so ruthlessly carried out that but few remain except in places difficult of access to the Indian in his fragile canoe. Deprived thus of the warmth giving fatty substance to which he had been accustomed, his system has become impoverished, and less able to resist the hostilities of the climate, and the hardships of his life"* (Stirling, 1891).

Nevertheless, we might consider two important distortions of modern-contemporary stranding records in relation to archeological contexts: first, and most evident, industrial hunting activities which may have significantly altered cetacean species' distributions and abundances (Clapham et al., 2008; Scott Baker and Clapham, 2004); secondly, the difficulty in accessing the Península Mitre (including the Southern Atlantic Coast of Isla Grande de Tierra del Fuego and the vicinity of Moat Channel and Beagle Channel). Península Mitre is essentially inhabited, and without roads. Therefore, it is probable that some strandings in this area have not been documented. The ethnographic accounts (primarily from the personal diaries of members of the Mission of the South American Missionary Society, established in Ushuaia in 1869) also recorded historic cetacean strandings but the species identifications are often imprecise or unreliable. Thus, biomolecular analysis of archeological remains provides an additional method to corroborate and enhance (pre-)historic records of whale distributions in this area.

#### 4.3. Archeological implications for HGF aggregation

The biomolecular identifications open several new lines of discussion in relation to the role of cetaceans in HGF societies, and the material correlates of aggregation processes. Initially, we had proposed that correlates of an aggregation around a stranded whale would include the predominant exploitation of a single cetacean species at the Lanashuaia locality, the identification of the same cetacean species in neighboring middens, and shared social practices (food sharing, shared activity patterns).

Previous morphological analysis of whale elements from both sites supported the possibility of an aggregation exploiting a young minke whale. Our biomolecular analyses, however, detected no minke whales. Unexpectedly, we revealed the presence of five other cetacean species: Southern bottlenose whale, Southern right, blue, humpback, and sei, providing new evidence for the range of cetacean species exploited by the Yámana. Although the exploitation of humpback whale and right whale had been proposed based on morphological classifications (Piana, 2005), blue whale and bottlenose whale had not been previously identified in the archeological record of the Beagle Channel. In general, these taxa display a quite homogeneous distribution between archeological layers, with no particular species dominating the assemblage (Fig. 7).

In light of modern ecological records and ethnographic sources, it is highly probable that all the bones were obtained from stranded whales or beached whale carcasses. While written sources provide frequent examples of whale strandings, evidence of active whale hunting is scarce and generally related to sick or wounded animals that had been deliberately driven ashore by Yámana people (Gusinde, 1937; Lothrop, 1928). The taxonomic diversity of cetaceans present in Lanashuaia thus indicates that strandings may not have been particularly rare occurrences or restricted to particular cetacean species.

The biomolecular results do not provide conclusive data concerning the hypothesis of social aggregation. The range and distribution of identified taxa contradicts our initial expectations in relation to whale bones as a marker of an aggregation episode. Nevertheless, data obtained by molecular methods do not allow us to completely refute the original supposition for three related reasons.

First, there are few morphological markers on the bones to indicate whether the cetacean resources procured at Lanashuaia were for

nutritional consumption or other purposes. Both the utility of whale bones as a raw material for tool-making as well as the ease with which the meat and fat can be extracted without leaving observable traces, make it difficult to confirm to what extent these species were exploited as food (Mulville, 2002). According to early 20C historical sources, beaked whales (such as Southern bottlenose) were avoided by Beagle Channel inhabitants because their fat had purgative effects (Gusinde, 1937, pp. 501, 559, and 567); these types of food taboos can be difficult to resolve in the archeological record.

Second, although only five bones were tested from the much larger whale assemblage at Lanashuaia I, the same species (blue, humpback, right) were observed, and in the case of blue whale, the same D-loop haplotype was present in the adjacent site. Thus, the similarity in the distribution of species may suggest shared social practice in the distribution of whale resources.

Third, the distance between the two annular structures is minimal (6–8 m), and so a great part of the carcass could lie in a specific place (e.g. Lanashuaia I) and meat and fat could have been distributed between several dwellings. The identification of similar species and shared mtDNA haplotypes, however, are not sufficient for confirming the exploitation of the same whale individual. More discriminating microsatellite analysis would be required in order to confirm the distribution of individual whale products between sites. Although for some species represented (such as the blue whale) our biomolecular data is consistent with interactions between different familiar units and the distribution of stranded whale products, more refined genetic analysis would be required to prove shared social practice at the Lanashuaia locality (Martial, 1888: 180).

These biomolecular results must be considered in combination with other evidence from the archeological sites: in both cases, the vast portion of identified bones are from the deepest layers of the archeological sites or in direct contact with the ancient beach, and consequently relate to the earliest occupation of the sites. Likewise, radiocarbon dates suggest a remarkable chronological proximity and the results of oxygen isotope analysis of Lanashuaia I and II indicate, in both cases, a human presence in winter or surrounding seasons. Although the biomolecular results suggest that whale bones are problematic as markers of an aggregation event, they shed light on the complexity of cetacean exploitation by HGF groups, and provide a scaffold for more refined hypotheses and future analyses.

## 5. Conclusions

Analyses of bone samples from the Lanashuaia locality highlights the value of biomolecular methods to distinguish morphologically unidentifiable bone fragments and to discriminate cetacean species within southern hemisphere archeological contexts. In particular this study exemplifies the relative strengths of collagen and DNA-based methods for paleoenvironmental research: ZooMS as a rapid biomolecular and taxonomic screening tool and DNA to provide robust species or haplotype identifications.

Although accurate biomolecular identifications were obtained for the cetacean remains, the evidence presented here is insufficient to fully evaluate the hypothesis of social aggregation in the HGF societies of Tierra del Fuego. Cetaceans were crucial for fueling historic aggregation events and for providing an economic incentive for congregating people in temporary concentrations. However, due to the lack of diagnostic butchering traces, the multiple uses of whale remains, and the transportation practices related to fat and meat, whale bones may not necessarily provide conclusive evidence for aggregation processes.

Our new understanding of cetacean exploitation in the region, including the taxonomic diversity of exploited species provides a new lens through which to explore the question of social aggregation and its potential material correlates. These biomolecular methods may also provide a new avenue for exploring the use of particular cetacean species as food and/or raw material sources. Previous studies have noted

the significance of bone mechanical properties for the manufacture of specific tools (Scheinsohn and Ferretti, 1995). Considering the minute quantities of bone required for accurate biomolecular species identification, ZooMS and DNA analysis both provide potential techniques for examining the relationship between raw materials and manufacturing techniques in HGF societies.

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.jasrep.2015.10.025>.

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